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(71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES NATIONAL INSTITUTES OF HEALTH [US/US]; Office of Technology Transfer, Box OTT, Bethesda, MD 20892 (US).

(72) Inventors: REITZ, Marvin, S. ; 17833 Bowie Road, Derwood, MD 20355 (US). WATERS, David, J. ; 10513 Grindstone Run Road, Myersville, MD 21773 (US). BLATTNER, William ; 5006 Wickett Terrace, Bethesda, MD 20814 (US). WILSON, Carolyn, A. ; 2002 N. Kenmore Street, Arlington, VA 22207 (US).

(74) Agents: SCOTT, Watson, T. et al.; Cushman, Darby & Cushman, Suite 900, 1100 New York Avenue N.W., Washington, DC 20005 (US).

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(54) Title: METHOD OF DETECTING INFECTIOUS VIRUS STRAINS

(57) Abstract

The present invention relates to a new assay system which can be used to screen for and to quantitate infectious retroviral strains. In particular, the present invention relates to a simple and rapid colorimetric transactivation assay for the detection of HIV-1, HIV-2 and SIV using HeLa cells containing CD4 and a reporter gene, for example β-galactosidase, that is dependent on transactivation by the infecting virus for expression.

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METHOD OF DETECTING INFECTIOUS VIRUS STRAINS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a new assay system which can be used to screen for and to quantitate infectious retroviral strains. In particular, the present invention relates to a simple and rapid colorimetric transactivation assay for the detection of HIV-1, HIV-2 and SIV using HeLa cells containing CD4 and a reporter gene, for example, ß-galactosidase, that is dependent on transactivation by the infecting virus for expression.

Background Information

Rocancourt et al reported the construction of an expression plasmid and the use of that plasmid in a detection system based on the trans-activation of the HIV-1, LTR and expression of ß-galactosidase *J. Virology* 64:2660-2668 (1990). This expression plasmid contained the early SV40 promoter and enhancer sequences internal to the HIV-1 LTR. For this reason, Rocancourt et al reported low basal levels of ß-galactosidase activity in exponentially growing cells, although this disappeared in their confluent cultures.

The adherent CD4 containing cells (preferably HeLaT4 cells) used to express the plasmid HXB2 Flacz of the present invention have been subcloned and selected on the basis of having no basal activity under any circumstances. This makes it p ssible t use the assay system of the pres nt inv ntion t titer or d t ct low lev ls of viruses. Similarly, this selection distinguishes

the cell line of the present invention from the rabbit cell line described by Roberts and Blair (*Antiviral Chemistry and Chemotherapy* 1:139-148 (1990)). Roberts and Blair developed a rabbit cell line expressing a construct analogous to HXB2Flacz (HIV-1 LTR and ß-galactosidase), but these authors screened for a cell line that had detectable levels of basal ß-galactosidase activity so that basal levels could be compared to those observed after activation by HIV-1 tat or other trans-activator of the HIV-1 LTR.

Although HIV infectivity assays that use adherent HeLa cells expressing the CD4 receptor have been reported by others, the quantitation of non-fusogenic virus variants with these cells has proved difficult. Thus, it is clear that a need exists for a simple, rapid and sensitive colorimetric transaction assay that detects and quantitates infectious HIV strains.

The present invention provides for such an assay. By transfecting an expression plasmid HxB2-LaczF into HeLa cells with the CD4 receptor and selecting out those cells capable of expressing ß-galactosidase following transactivation by HIV, the present invention makes it possible to detect infection of a cell by a single virion of HIV-1 or HIV-2 where the infected cells can be detected as blue cell clusters or syncytium.

Similar transactivation assays for expression of prokaryotic enzymes such as chloramphenicol acetyl transferase (CAT) in eukaryotic cells have been developed by others for HIV detection; however, these assay require the use of radioactive label and autoradiography to obtain results. Compared to CAT assays, the present invention provides for the measurement of HIV transactivated marker gene expression in a faster, equally sensitiv and more conv ni nt mann r.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a fast, sensitive and convenient assay for screening and quantitating infectious viral strains.

It is another object of the invention to provide a cell line for use in the above-described assay system that permits detection of infection by as little as a single virion.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the expression plasmid HXB2Flacz (Spe-Xho). The plasmid sequences, derived from pSP65 gpt are represented as jagged black lines; the HIV LTR's as rectangles with hash marks; HIV coding regions as rectangles with dots, and the ß-galactosidase coding region an empty rectangle.

Figure 2 represents a clone of the HeLaT4 lacz cells challenged with HIV-1/IIIB. ß-galactosidase positive (blue) cell clusters may still be seen in the 10^4 virus dilution.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a sensitive and rapid colorimetric transactivation bioassay that detects infectious retroviral strains, for example, HIV-1 and HIV-2 strains using cells, for example, HeLa cells, containing CD4 and a

reporter gene, for example the ß-galactosidase (Lacz) gene.

In a preferred embodiment, the present invention relates to an assay that may be simply performed by utilizing adherent CD4 containing HeLa cells (preferably, HeLaT4 cells) that are transfected with the expression plasmid HxB2-Lac2F. Expression of the fusion protein ß-galactosidase by the transfected HeLaT4-cells is dependent on transactivation by the infecting virus, for example by the tat gene. When these construct containing cells are infected with the virus, the infected cells can be detected and quantified, for example, as clusters of blue cells or syncytia. A variety of substrates are available for the detection of ß-galactosidase, microscopically as fixed blue cells, by fluorescent excitation and measurement in a fluorometer, or viably sorted by FACS.

It should also be noted that the present invention relates to the transactivated expression of ß-galactosidase, detected by the 5-bromo-4-chloro-3-indolyl-ß-D-glucopyranoside (X-Gal) histochemical assay which is scored macroscopically and microscopically at low magnification. Virus titers obtained using the HeLaT4-Lacz cells are comparable to those obtained by endpoint dilution using HuBPL's and indirect assays. Wild-type nonfusogenic HIV-1 isolates may by titered with the assay of the present invention.

The assay system provided by the present invention results in the elimination of the need for an assay using endpoint dilutions to yield quantitative results where the virus is detected with time consuming and expensive indirect assays such as reverse transcriptase or p-24 antigen capture.

In particular, th present invention provid s for an assay system that exhibits a s nsitivity similar to that obtained using peripheral blood lymphocyt s (PBL's) and will speed and simplify a variety of studies requiring the assay of live virus, agents or molecular constructs capable of activating the HIV-1 LTR. This adherent cell, nonradioactive, 72 hour assay system can be done in 96 micro-well plates.

In a further preferred embodiment, the present invention relates to a DNA construct, expression plasmid HxB2-LaczF, comprising a DNA segment encoding a HIV gag-ß-galactosidase fusion protein and a vector. The HxB2-LaczF construct contains both the 5' and 3' HIV LTRs where expression of the fusion protein is dependent upon activation of the HIV-LTR by a transactivator, for example, tat.

In yet another embodiment, the present invention relates to host cells, for example HeLa cells with the CD4 receptor, that produce a marker, such as ß-galactosidase, following transactivation by HIV-1, HIV-2, SIV or biologically active molecular constructs. In the Examples that follow, the DNA construct, HxB2-LaczF, provided herein which expresses bacterial ß-galactosidase as a HIV-gag fusion protein, was transfected into CD4 + HeLa cells with the gpt selectable marker.

In a further embodiment, the present invention relates to the deposited cell lines, HeLaT4-Lacz clone F-7, 2≠10 and clone H-10, 3≠2 given the accession numbers, 10765 and 10766, respectively. The cell lines were deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on June 21, 1991.

In yet another embodiment, the present invention relates t a diagnostic assay to

quantitate the infecting titers of fusigenic and nonfusigenic strains of cell free virus.

Yet another embodiment of the present invention relates to a detection assay suitable for use in an infectious center assay that quantitates the number of virus infected cells from patient or animals. For example, the effectiveness of a drug or vaccine in eliminating or reducing the number of circulating cells carrying the virus in AIDS patients can be ascertained using the assay of the present invention.

In another embodiment, the present invention relates to a virus neutralization assay to quantitate virus neutralizing antibody in serum obtained from patients, vaccine recipients or animals. Using the transactivation assay of the present invention, virus strain variations can be checked and peptide competition neutralization assays can be made rapidly and easily.

Yet another embodiment of the present invention relates to a detection method using the transactivation assay of the present invention that may provide a rapid and sensitive means for the large scale screening and detection of compounds that either prevent virus infection or suppress its replication following infection.

In a further embodiment, the present invention relates to a diagnostic method using the transactivation bioassay. Specific drug resistant viruses can be selected from patients using the assay of the present invention followed by FACS to retrieve the cells containing the virus.

Another embodiment of the present invention relates to a diagnostic method to identify any cell transfected with molecular constructs which may transactivate HIV-1. The transactivation bioassay provides a simple and rapid method to

identify and separate by FACS any cells transfected with molecular constructs which can transactivate the HIV-1 LTR, thus eliminating the need for drug selection.

In a further embodiment, the present invention relates to a novel method to isolate new or wild type lentivirus from clinical samples. The HxB2-LaczF construct-containing HeLa cells provided in the present invention can be used in the transactivation assay, also provided in the present invention, to activate expression of the reporter gene, ß-galactosidase when infected with a new or wild type lentivirus. Transactivated cells expressing ß-galactosidase can be directly removed from the monolayer by pipette, cloned and sequenced by PCR.

The following examples are given to further illustrate the present invention without being deemed limitative thereof.

EXAMPLES

The following materials/protocols are referred to in the Examples that follow.

CELLS AND VIRUSES

HeLaT4-cells, human cervical cancer cells, which express the CD4 molecule obtained from Dr. Richard Axel, Columbia University were grown in Dulbeccos Modified Eagles Medium (DMEM) with 10% fetal bovine serum (FBS). CEM cells obtained from Dr. Robert Gallo were cultivated in RPMI 1640 medium with 10% FBS. HIV-1 virus strains IIIB, RF and MN, as well as HIV-2 strain NIH_z were propagated in CEM cells. Cell-free virus was harvested 72 hours post infection, aliquoted and stored at -85°C until used.

INFECTIVITY ASSAY

HeLaT4-Lavz cells were seeded into 16-well (NUNC) chamber slides or 96-well (Costar) tissue culture plates at 1.5 X 10^4 cells/well in a 200 ml volume of DMEM, 10% FBS. After 48 hours incubation at 37°C in 5% CO_2 the media was removed and replaced with 200 µl of diluted virus stock. At this time, 50-60% of the well was covered by adherent cells. Dilutions of virus stocks were made with DMEM, + 10% FBS on ice. Each virus dilution was plated in duplicate and the slide or plate incubated at 37°C in 5% CO_2. After 24 hours of incubation, the inoculum was discarded and replaced with 300 µl of DMEM, 10% FBS and incubation of the slide or plate continued for an additional 48 hours at 37°C, 5% CO_2.

INFECTIVITY NEUTRALIZATION ASSAY

Slides and plates were prepared as described above. Human sera from HIV infected individuals were diluted in DMEM without FBS. Cell-free HIV-1 stock virus was diluted 1:50 in DMEM and equal columns added to each of the serum dilutions to yield a final virus concentration of 2.6 X 10^3 PFU/200 µl. Following 1 hour of incubation at 37°C with occasional agitation, 200 µl of each virus-serum dilution was plated onto 48 hour old HeLaT4-LaczF cells in either chamber slides or 96-well plates. Following overnight incubation at 37°C with 5% CO_2, an additional 200 µl of DMEM, 10% FBS was added to each well, replaced at 37°C and incubation continued for an additional 48 hours prior to development.

ß-GALACTOSIDASE ASSAY (X-GAL)

At 72 hours post-infection the cells were rinsed once with cold PBS after removal of the cover and superstructure of the chamber slide. The cells were fixed with a cold 2% glutaraldehyde and 2% formaldehyde, PBS solution for 5 minutes followed by a 5 minute cold PBS wash. Excess liquid was removed and 120 µl of X-Gal reagent (20 mg of 5-bromo-4-chloro-3-indolyl-ß-D-glucopyranoside, 1.0 ml of 5 mM ferricyanide, 1.0 ml of 5mM ferricyanide, and 40 µl of 2 mM $MgCl_2$ in a total PBS volume of 20 mls) was added to each well, incubated at 37°C overnight and the slides scored macro- and microscopically.

EXAMPLE 1

CONSTRUCTION OF HXB2FLACZ (SPE-XHO)

The expression plasmid was derived from a three-way ligation of the following fragments: (1) pHXB-2D digested with Xho I, filled in the T4 DNA polymerase, and digested with SpeI; (2) pl203 digested with Eco RI, filled in with T4 DNA polymerase, and digested with BamHI; and (3) a linker fragment consisting of two annealed oligonucleotides with the following sequence:

5' CTAGTGCGGCCGCG 3'

3' ACGCCGGCGCTAG 5'

The annealed linker fragment when ligated correctly to the other two fragment carries the palindromes recognized by three restriction enzymes: SpeI, NotI and BamHI; it also maintains an open reading frame between the HIV-1 gag gene and lacz. The resulting plasmids were screened first by restriction analysis (SpeI and ClaI-shown in Figure 1), and the presence of the linker was confirmed by dideoxy sequencing.

EXAMPLE 2

EXPRESSION OF HXB2FLACZ (SPE-XHO)

The expression plasmid was transfected into HeLaT4 cells by calcium phosphate mediated DNA transfection. Twenty-four hours post-transfection the medium was changed. Forty-eight hours post-transfection the cells were seeded at approximately 5 X 10^5 cells/10 cm plate. After 24 hours the media was changed to selective media (DMEM supplemented with 250 µl/ml mycophenolic acid and 250 µg/ml xanthine) in order to select for cells which had taken up and expressed the gpt marker on the plasmid. After three weeks of selection, colonies were transferred to 12-well plates using cloning cylinders. Expression of the gag-ß-galactosidase fusion protein was assessed after transfection with pCV-1 (HIV-1 tat). Transfected and untransfected clones were histochemically stained for expression of ß-galactosidase. One of these clones with one in 500 cells positive for expression of ß-galactosidase was subcloned in 96-well plates. Wells containing single cells were identified and marked. After the cell in each marked well had formed a colony they were split between two 96-well plates with the cells in one plate challenged by HIV-1/IIIB. Twelve-wells containing cells exhibiting the greatest expression of ß-galactosidase following virus challenge were identified and the corresponding uninfected cells from the sister plate were single cell cloned a second time. Cells from each of these twelve wells were seeded into 96-well plates at a cell concentration to yield a single cell in each well. Wells containing single cells were identified, marked and handled as d scribed above. Twenty clon s derived from single cells wer identified

which possess d high levels of virus inducible ß-galactosidase activity. The point of optimum HIV virus induced ß-galactosidase production and tit r was 72 hours post inf ction. Figur 2 is a representative clone of the HeLaT4 lacz cells challenged with HIV-1/IIIB. ß-galactosidase positive (blue) cell clusters can still be seen in the 10^4 virus dilution. Virus stocks of HIV-1 strains IIIB,RF and MN were titered by ß-galactosidase induction using the HeLaT4-Lacz cells. The resulting titers were comparable to these titers previously obtained using human peripheral blood lymphocytes (HuPBL's). The HeLaT4-Lacz cells can be used to determine the amount of virus neutralizing antibody in serum samples in virus neutralization assays.

* * * * * *

All publication mentioned hereinabove are hereby incorporated by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

What is Claimed is:

1. A bioassay for the detection of a retrovirus in a sample comprising the steps of:

i) contacting said sample with a construct-containing HeLa cell, said construct comprising an expression reporter gene, under conditions such that said cell is infected with said retrovirus, and

ii) detecting infection of said cell by the transactivated expression of the said reporter gene in said cell.

FIG. 1

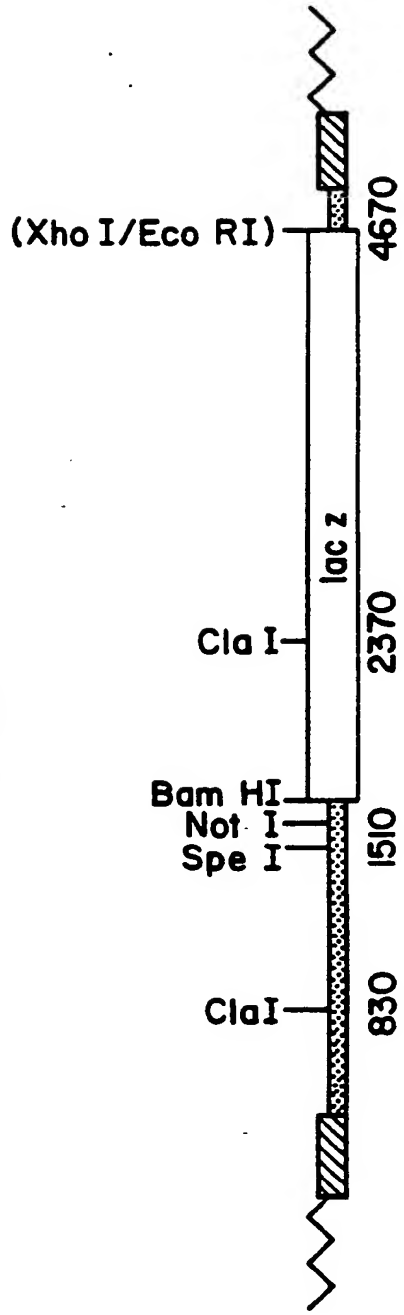
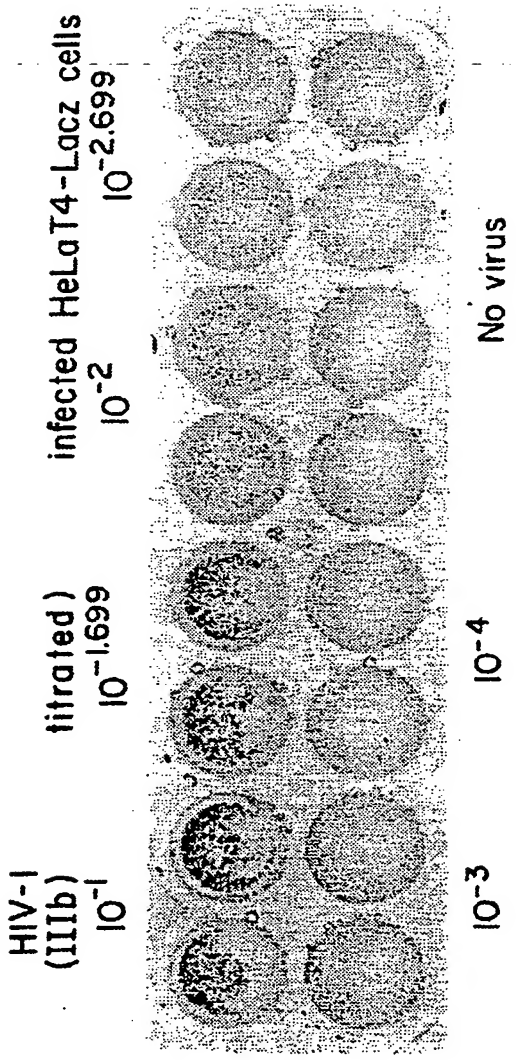


FIG. 2



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07386

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/70
US CL :435/5

According to International Patent Classification (IPC) r to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS 1985+, MEDLINE 1975+, NTIS, CA SEARCH, BIOTECHNOLOGY ABSTRACTS 1982+, WORLD PATENT INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Virology, Volume 64, Number 6, issued June 1990, D. Rocancourt et al, "Activation of β-Galactosidase Recombinant Provirus: Application to Titration of Human Immunodeficiency Virus (HIV) and HIV-Infected Cells," pages 2660-2668, see entire document, especially abstract.	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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